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TITLE OF THE INVENTION

GENE PARTICIPATING IN ACETIC ACID TOLERANCE, ACETIC ACID BACTERIA BRED USING THE GENE, AND PROCESS FOR PRODUCING VINRGAR WITH THE USE OF THE ACETIC ACID BACTERIA

Technical Field to Which the Invention Pertains

The present invention relates to genes that encode protein having a function of enhancing acetic acid tolerance derived from microorganisms, microorganisms whose copy numbers of the genes are amplified, particularly acetic acid bacteria which belong to the genus *Acetobacter* or the genus *Gluconacetobacter*, and a method of efficiently producing vinegar containing acetic acid at a high concentration by using these microorganisms.

Prior Art

Acetic acid bacteria are microorganisms widely used in producing vinegar, and particularly acetic acid bacteria which belong to the genus *Acetobacter* or the genus *Gluconacetobacter* are utilized for industrial acetic acid fermentation.

In acetic acid fermentation, ethanol in medium is oxidized to acetic acid by acetic acid bacteria, and acetic acid resultantly accumulates in the medium, but acetic acid is inhibitive for acetic acid bacteria as well, and growth ability and fermentation ability of acetic acid bacteria gradually decrease as the concentration of acetic acid in the medium increases.

Accordingly, it is required in acetic acid fermentation that growth ability and fermentation ability do not decrease even at higher concentration of acetic acid, i.e. development of acetic acid bacteria having strong acetic acid tolerance is required. It has been attempted as one of the means that genes participating in acetic acid tolerance (acetic acid resistance-genes) are cloned and acetic acid bacteria are bred and improved by using the acetic acid resistance-genes.

As for findings relating to acetic acid resistance-genes of acetic acid bacteria heretofore, three genes (aarA, aarB, and aarC) clustering have been cloned as complementary genes which can restore the acetic acid sensitive mutants of acetic acid bacteria belonging to the genus *Acetobacter*, to the original tolerance (see non-patent literature 1, for instance).

Among them, it was presumed that aarA gene encodes citric acid synthase and aarC gene encodes enzyme relating to assimilation of acetic acid, while the function of aarB gene has been uncertain (see non-patent literature 2, for instance).

In the transformants obtained by transformation with cloned gene fragment containing these three acetic acid resistance-genes on multi-copy plasmid to *Acetobacter aceti* subspecies *xylinum* IF03288 strain, the level of improvement of acetic acid tolerance was only low, and it was uncertain if ability of these three acetic acid resistance-genes in actual acetic acid fermentation was improved or not (see non-patent literature 1, for instance).

Meanwhile, there has been a disclosed example in which improvement of the final acetic acid concentration in acetic

acid fermentation was confirmed by introducing gene encoding membrane-bound aldehyde dehydrogenase (ALDH) cloned from acetic acid bacteria into acetic acid bacteria (see non-patent literature 2, for instance). However, since ALDH is not an enzyme directly participating in acetic acid tolerance but that having a function of oxidizing aldehyde, it could not be concluded whether gene encoding ALDH was the exact acetic acid resistance-gene.

Patent Literature 1

Japanese Laid-Open Patent Application No. 1991-219878

Patent Literature 2

Japanese Laid-Open Patent Application No. 1990-2364

Patent Literature 3

Japanese Laid-Open Patent Application No. 1985-9489

Patent Literature 4

Japanese Laid-Open Patent Application No. 1985-9488

Non-patent Literature 1

Journal of Bacteriology, vol. 172, 2096-2104, 1990

Non-patent Literature 2

Journal of Fermentation and Bioengineering, vol. 76, 270-275, 1993

Non-patent Literature 3

Applied of Environment and Microbiology, vol. 55, 171-176, 1989

Non-patent Literature 4

Agricultural and Biological Chemistry, vol. 52, p. 3125-3129, 1988

Non-patent Literature 5

Agricultural and Biological Chemistry, vol. 49, p. 2091-2097,

1985

Non-patent Literature 6

Bioscience, Biotechnology and Biochemistry, vol. 58, p. 974-975, 1994

Problem to be Solved by the Invention

As mentioned above, no example that has elucidated acetic acid tolerance of acetic acid bacteria on genetic level and has succeeded in the development of practical acetic acid bacteria having high acetic acid tolerance has been reported heretofore. However, development of acetic acid bacteria superior in acetic acid tolerance would allow the performance of acetic acid fermentation at higher concentration than that conventional one and efficient production of acetic acid at a high concentration and vinegar at a higher concentration. Therefore, the present inventors attempted again to elucidate the improvement of acetic acid tolerance of acetic acid bacteria on genetic level.

As a result of consideration from various aspects, and from the view point that it was important to obtain novel acetic acid resistance-genes encoding proteins that have a function capable of improving acetic acid tolerance on practical level and to breed acetic acid bacteria having stronger acetic acid tolerance with the use of the obtained acetic acid resistance-genes, the present inventors have newly set novel technical tasks to furnish novel genes participating in acetic acid tolerance derived from microorganisms belonging to acetic acid bacteria, and to furnish a method of improving acetic acid tolerance of microorganisms by using the genes, particularly a method

of improving acetic acid tolerance of microorganisms belonging to acetic acid bacteria, further a method of efficiently producing vinegar having acetic acid at a high concentration by using the acetic acid bacteria whose acetic acid tolerance was improved.

Means for Solving Problems

The present inventors hypothesized that specific genes participating in acetic acid tolerance that do not exist in other microorganisms should exist in acetic acid bacteria capable of growing and fermenting even in the presence of acetic acid, and the present inventors obtained a novel concept that the use of these genes would allow improvement of acetic acid tolerance of microorganisms more than before, further it would allow the development of an efficient method of producing novel vinegar containing acetic acid at a high concentration which could not be conventionally obtained.

As for the conventional method of obtaining acetic acid resistance-genes, it was popular that genes which complement mutant of acetic acid bacteria with acetic acid sensitivity were cloned.

However, thinking that it was difficult to find acetic acid resistance-genes which were industrially useful by such a method, the present inventors have developed a method as that of finding acetic acid resistance-genes from acetic acid bacteria, in which chromosomal DNA library of acetic acid bacteria was constructed, this chromosomal DNA library was transformed into acetic acid bacteria, that can generally grow only under up to approximately 1% acetic acid and the genes capable of growing the strain in the presence of 2%

acetic acid as well, were obtained by screening.

According to the use of this method, the present inventors have succeeded for the first time in cloning novel acetic acid resistance-genes that have a function of improving acetic acid tolerance on practical level from acetic acid bacteria belonging to the genus *Gluconacetobacter* practically used in producing vinegar.

Brief Description of the Drawings

Figure 1

This is a schematic view showing a restriction enzyme map of a gene fragment (pP1) derived from *Gluconacetobacter entanii* cloned by using PstI, the location of acetic acid resistance-gene and the insert into pSPT.

Figure 2

This is a schematic view showing a restriction enzyme map of a gene fragment (pP2) derived from *Acetobacter aceti* cloned by using inverse PCR method, the location of acetic acid resistance-gene and an insert into pSPT2.

Figure 3

This is a figure showing the time course in cultivation of the transformant whose copy numbers of acetic acid resistance-gene derived from *Gluconacetobacter entanii* were amplified.

Figure 4

This is a figure showing the time course in acetic acid fermentation at higher temperature by the transformant whose copy numbers of acetic acid resistance-gene derived from *Gluconacetobacter entanii* were amplified.

Figure 5

This is a figure showing an amino acid sequence (SEQ. ID No. 2) of the protein deduced by an acetic acid resistance-gene derived from *Gluconacetobacter entanii*.

Figure 6

This is a figure showing an amino acid sequence (SEQ. ID No. 4) of the protein deduced by an acetic acid resistance-gene derived from *Acetobacter aceti*.

Figure 7

This is a figure showing primer 1.

Figure 8

This is a figure showing primer 2.

Figure 9

This is a figure showing primer 3.

Figure 10

This is a figure showing primer 4.

Figure 11

This is a figure showing primer 5.

Figure 12

This is a figure showing primer 6.

The obtained acetic acid resistance-genes showed homology with protein referred to as serine palmitoyltransferase that catalyze the first process of sphingolipid synthesis found in *Sphingomonas* and the like, and they were presumed as genes that encode serine palmitoyltransferases of acetic acid bacteria, as a result of homology search on DDBJ/EMBL/Genbank and SWISS-PROT/PIR.

However, the aforementioned gene of serine palmitoyltransferase from genus *Sphingomonas* was the only example in prokaryotes heretofore.

Further, one of the obtained serine palmitoyltransferase genes of acetic acid bacteria had 46.3% homology on amino acid sequence level with known serine palmitoyltransferase gene found in the genus *Sphingomonas* and it had approximately 25% homology with that of mice, the ratio of which were so low that it was confirmed that it was similar to other serine palmitoyltransferase genes to some extent, but it was novel gene encoding the novel protein (it is sometimes referred to as protein SPT) specific to acetic acid bacteria.

In addition, in the transformants in which the genes were inserted to plasmid vectors and transformed into acetic acid bacteria and their copy numbers were amplified, remarkable improvement in acetic acid tolerance could be seen. When the transformants were subjected to aerobic cultivation in the presence of ethanol, we found that growth rate and production rate were improved as well as growth lag time was shortened, further final acetic acid concentration was remarkably improved as a result. The present inventors further have succeeded in determination of the nucleotide sequence and the deduced amino acid sequence of genetic DNA encoding thereof. According to these findings, we have completed the present invention.

Disclosure of the Invention

The embodiment of the present invention is as follows.

(1) A protein SPT described in following (A) or (B):

(A) A protein having an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing.

(B) A protein consisting of an amino acid sequence comprising

substitution, deletion, insertion, addition, or inversion of one or several amino acids in an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing and having a function of enhancing acetic acid tolerance.

(2) A DNA of a gene encoding the protein SPT described in following (A) or (B):

(A) A protein having an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing.

(B) A protein consisting of an amino acid sequence comprising substitution, deletion, insertion, addition, or inversion of one or several amino acids in an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing and having a function of enhancing acetic acid tolerance.

(3) The DNA of a gene according to (2), that is a DNA described in following (a) or (b):

(a) A DNA that comprises a nucleotide sequence consisting of nucleotides 187 to 1386 shown in SEQ. ID No. 1 in the sequence listing within the nucleotide sequence.

(b) A DNA that hybridizes with a probe comprising a nucleotide sequence consisting of nucleotides 187 to 1386 shown in SEQ. ID No. 1 in the sequence listing within the nucleotide sequence or a part thereof under a stringent condition, and encodes protein having a function of enhancing acetic acid tolerance.

(4) A protein SPT2 described in following (A) or (B):

(A) A protein having an amino acid sequence shown in SEQ. ID No. 4 in the sequence listing.

(B) The protein SPT2 consisting of an amino acid sequence comprising substitution, deletion, insertion, addition, or inversion of one or several amino acids in an amino acid

sequence shown in SEQ. ID No. 4 in the sequence listing and having a function of enhancing acetic acid tolerance.

(5) A DNA of a gene encoding the protein SPT2 described in following (A) or (B):

(A) A protein having an amino acid sequence shown in SEQ. ID No. 4 in the sequence listing.

(B) The protein SPT2 consisting of an amino acid sequence comprising substitution, deletion, insertion, addition, or inversion of one or several amino acids in an amino acid sequence shown in SEQ. ID No. 4 in the sequence listing and having a function of enhancing acetic acid tolerance.

(6) The DNA of a gene according to (5), that is a DNA described in following (A) or (B):

(A) A DNA that comprises a nucleotide sequence consisting of nucleotides 110 to 1321 shown in SEQ. ID No. 3 in the sequence listing within the nucleotide sequence.

(B) A DNA that hybridizes with a probe generated from a nucleotide sequence consisting of nucleotides 110 to 1321 shown in SEQ. ID No. 3 in the sequence listing within the nucleotide sequence or a part thereof under a stringent condition, and encodes protein having a function of promoting growth rate.

(7) Microorganisms wherein acetic acid tolerance thereof is enhanced by amplifying an intracellular copy number of the DNA according to any one of said (2), (3), (5), or (6).

(8) The microorganisms according to said (7), wherein the microorganisms are acetic acid bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter*.

(9) A method of producing vinegar, wherein microorganisms having alcohol oxidation ability among the microorganisms

according to said (7) or (8), are cultured on medium containing alcohol and they produce and accumulate acetic acid in the medium, and a novel vinegar obtained therefrom whose acetic acid content is high (10-17.5%).

(10) A recombinant plasmid pUSPT (FERM BP-7932) including the DNA according to said (2) or (3), or a recombinant plasmid pUSPT2 (FERM BP-8304) including the DNA according to said (5) or (6).

(11) A recombinant plasmid comprising a DNA fragment having at least a nucleotide sequence shown in SEQ. ID No. 1 or 3, for instance plasmid pSPT or plasmid pSPT2 obtained from inserting these DNA fragments to an acetic acid bacteria-Escherichia coli shuttle vector (a multi-copy vector) pMV24, and/or a transformant which is obtained by introducing these plasmids pSPT and pSPT2 into Acetobacter aceti No. 1023 (FERM BP-2287) or Acetobacter altoacetigenes MH-24 strain (FERM BP-491).

By the present invention, tolerance against acetic acid can be provided toward microorganisms. Moreover, in microorganisms having alcohol oxidation ability, particularly in acetic acid bacteria, tolerance against acetic acid can be remarkably improved and the ability of efficiently accumulating acetic acid at a high concentration in the media can be provided.

Best Mode for Carrying out the Invention

The present invention will be explained in more detail.

(1) The DNA of the present invention

The DNA of the present invention comprises homology with serine palmitoyltransferase of the genus *Sphingomonas*

to some extent, and a nucleotide sequence that can encode protein having a function of improving acetic acid tolerance and comprising an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing, and it comprises regulating elements and the structural gene.

The DNA of the present invention can be obtained from chromosomal DNA of *Gluconacetobacter entanii* as follows.

First, chromosomal DNA library of *Gluconacetobacter entanii*, for instance *Acetobacter altoacetigenes* MH-24 strain (deposited as FERM BP-491 with International Patent Organism Depositary) is prepared. Meanwhile chromosomal DNA is obtained by the method disclosed in patent literature 3.

Next, a chromosomal DNA library is prepared in order to isolate acetic acid resistance-genes from the obtained chromosomal DNA. To begin with, the chromosomal DNA is partially digested by suitable restriction enzymes to obtain various DNA fragment-mixtures. Various kinds of restriction enzymes can be used by adjusting cleavage reaction time and the like to control the extent of cleavage. For instance, chromosomal DNA is digested by the restriction enzyme of *Sau3AI* at the 30°C or higher, preferably 37°C, at enzyme concentration of 1-10 units/ml for various time periods (1 min-2 h). Meanwhile, *PstI* was used in after-mentioned Examples.

Then digested fragments of chromosomal DNA are ligated to vector DNA that can replicate autonomously in acetic acid bacteria to prepare recombinant DNA. Specifically, this vector DNA is completely digested by the action of a restriction enzyme which yields a terminal nucleotide

sequence complementary to the PstI restriction enzyme used for digesting of chromosomal DNA, for instance PstI, under the condition at the temperature of 30°C and enzyme concentration of 1-100 units/ml for 1 h or longer.

Subsequently, the obtained chromosomal DNA fragment-mixtures in the above-mentioned manner are mixed with the digested vector DNA, then recombinant DNA is obtained by the action of T4DNA ligase to the mixture under the condition at the temperature of 4-16°C, at enzyme concentration of 1-100 units/ml for 1 h or longer, preferably 6-24 h.

Using the obtained recombinant DNA, acetic acid bacteria generally incapable of growing in the presence of acetic acid at higher than 1% concentration on agar media, for instance *Acetobacter aceti* No. 1023 strain (deposited as FERM BP-2287 with International Patent Organism Depositary) are transformed, and cultivated on agar media containing 2% acetic acid. DNA fragments including acetic acid resistance-genes can be obtained by cultivating there generated colonies into liquid media, then recovering plasmids from the obtained bacterial cells.

As for the DNA of the present invention, DNA comprising a nucleotide sequence shown in SEQ. ID No. 1 or 3 in the sequence listing is specifically exemplified, among which the nucleotide sequence consisting of nucleotides 187 to 1386 shown in SEQ. ID No. 1 within the nucleotide sequence or nucleotides 110 to 1321 shown in SEQ. ID No. 3 within the nucleotide sequence is coding region.

As the result of homology search on DDBJ/EMBL/Genbank and SWISS-PROT/PIR about the nucleotide sequence shown in

SEQ. ID No. 1, an amino acid sequence shown in SEQ. ID No. 2 (Figure 3: corresponding to nucleotides 187 to 1386), the nucleotide sequence shown in SEQ. ID No. 3 or an amino acid sequence shown in SEQ. ID No. 4 (Figure 4: corresponding to nucleotides 110 to 1321), the nucleotide sequence shown in SEQ. ID No. 1 or the amino acid sequence shown in SEQ. ID No. 2 showed 46.3% homology with SPT1 gene of *Sphingomonas paucimobilis*, while showed 26.3% and 24.8% homologies with LCB1 gene and LCB2 gene of mice, respectively on amino acid sequence level, and the genes were presumed as those encoding serine palmitoyltransferases, but each of their homologies was as low as 50% or less, so that it was apparent that this was novel and different from these genes.

Further, the nucleotide sequence shown in SEQ. ID No. 2 or the amino acid sequence shown in SEQ. ID No. 4 showed 46.7% homology with SPT1 gene, while showed 22.6% and 19.8% homologies with LCB1 gene and LCB2 gene of mice, respectively on amino acid sequence level, and the genes were presumed as those of encoding serine palmitoyltransferases, but each of their homologies was as low as 50% or less and it was apparent that this was novel and different from these genes.

Meanwhile, it has been unknown at all that aforementioned SPT gene and the like participate in acetic acid tolerance.

Further, the DNA of the present invention was identified that it is a novel gene having a function of enhancing the acetic acid tolerance which is different from previously obtained acetic acid resistance-genes (aarA, aarB and aarC) of acetic acid bacteria, ADH gene having a function of enhancing acetic acid tolerance or the like.

As the nucleotide thereof was revealed, for instance the DNA of the present invention can also be obtained by polymerase chain reaction (PCR reaction) using genomic DNA of the acetic acid bacteria *Gluconacetobacter entanii* as a template and oligonucleotide synthesized based on the nucleotide sequence as a primer, or by hybridization using oligonucleotide synthesized based on the nucleotide sequence as a probe, as well.

As for the synthesis of the oligonucleotide, it can be synthesized, for instance using commercially available various DNA synthesizers in the conventional manner. In addition, PCR reaction can be performed in the conventional manner using Thermal Cycler Gene Amp PCR System 2400 (Applied Biosystems) and Taq DNA polymerase (Takara Shuzo Co., LTD.), KOD-Plus- (TOYOBO Co., LTD.) and the like.

As for the DNA encoding the protein having a function of enhancing acetic acid tolerance of the present invention, it may be a DNA encoding protein where one or several amino acid is deleted, substituted, inserted or added at one or several sites, as long as the function of enhancing acetic acid tolerance of the encoded protein is not impaired.

The DNA encoding a protein substantially identical to such protein having a function of enhancing acetic acid tolerance, can also be obtained by modifying the nucleotide sequence so that amino acid at a particular site is deleted, substituted, inserted or added by site-directed mutagenesis, for instance. Further, the modified DNA such as aforementioned one can also be obtained by conventionally known mutagenesis treatments.

In addition, as an amino acid sequence of protein and

a nucleotide sequence encoding thereof are generally known that they are slightly different among species, strains, mutants, and variants, the DNA encoding substantially identical proteins can be obtained from general acetic acid bacteria, among which, species, strains, mutants and variants of the genus *Acetobacter* or the genus *Gluconacetobacter*.

Specifically, DNA encoding a protein substantially identical to the protein can be obtained from acetic acid bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter*, acetic acid bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter* treated for mutagenesis, or natural mutants or variants thereof, by for instance, isolating DNA: hybridizing under a stringent condition with DNA comprising a nucleotide sequence consisting of nucleotides 187 to 1386 shown in SEQ. ID No. 1 in the sequence listing within the nucleotide sequence or with DNA comprising a nucleotide sequence consisting of nucleotides 110 to 1321 shown in SEQ. ID No. 3 in the sequence listing within the nucleotide sequence, and DNA: encoding protein having a function of enhancing acetic acid tolerance. The term stringent condition here is a condition in which so-called specific hybrid is formed while non-specific hybrid is not formed. Though it is difficult to quantify this condition clearly, if one example is taken, a condition in which DNA having high homology, for instance DNA having homology of 70% or more hybridizes, while DNA having homology lower than that does not hybridize, or a condition in which general washing is performed for hybridization, for instance the washing is performed with 0.1% SDS at the salt

concentration equivalent to 1 × SSC at 60°C, can be exemplified.

(2) The acetic acid bacteria of the present invention

The acetic acid bacteria of the present invention mean bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter*, or such bacteria, belonging to the genus *Acetobacter* or the genus *Gluconacetobacter* which are enhanced their acetic acid tolerance.

In bacteria belonging to the genus *Acetobacter*, *Acetobacter aceti* can be cited specifically, and *Acetobacter aceti* No. 1023 strain (deposited as FERM BP-2287 with International Patent Organism Depositary) can be exemplified.

Further, as for bacteria belonging to the genus *Gluconacetobacter*, *Gluconacetobacter entanii* can be cited, and *Acetobacter altoacetigenes* MH-24 strain currently deposited as FERM BP-491 with International Patent Organism Depositary can be exemplified.

Acetic acid tolerance can be enhanced, for instance, by amplifying intracellular copy numbers of acetic acid resistance-genes, or transforming bacteria belonging to the genus *Acetobacter* by using recombinant DNA obtained by ligated DNA fragments containing structural genes to promoter sequences that function efficiently in the bacteria belonging to the genus *Acetobacter*.

In addition, acetic acid tolerance can also be enhanced by replacing the promoter sequence of the genes on chromosomal DNA with other promoter sequence derived from microorganisms functioning efficiently in bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter*, for instance

such promoters of other than acetic acid bacteria including ampicillin-resistance gene for plasmid pBR322, kanamycin-resistance gene for plasmid pACYC177, and chloramphenicol-resistance gene for plasmid pACYC184, β -galactosidase gene and the like from *Escherichia coli*.

Amplification of intracellular copy numbers of the genes can be conducted by introducing multi-copy vectors retaining the genes into cells of bacteria belonging to the genus *Acetobacter*, i. e. it can be conducted by introducing plasmid, transposon and the like retaining the genes into cells of bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter*.

As for multi-copy vectors, pMV24 (see non-patent literature 3, for instance), pTA5001 (A), pTA5001 (B) (see patent literature 4, for instance) and the like can be exemplified, and pMVL1 which is a chromosome-integrative type vector (see non-patent literature 4, for instance) can also be cited. Further, as for transposons, Mu, IS1452 and the like can be exemplified.

Transformation of DNA into acetic acid bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter* can be performed by calcium chloride method (see non-patent literature 5, for instance), electroporation method (see non-patent literature 6, for instance) and the like.

Enhancement of acetic acid tolerance in acetic acid bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter* having alcohol oxidation ability in the above-described manner, enables increase of the amount of production and production efficiency of acetic acid.

(3) Method of producing vinegar

Bacteria belonging to the genus *Acetobacter* or the genus *Gluconacetobacter*, which are selectively enhanced their acetic acid tolerance by amplification of copy numbers of acetic acid resistance-genes in the above-described manner, and which have alcohol oxidation ability, are cultured on media containing alcohol, and acetic acid is produced and accumulated in the media. Vinegar can be thus efficiently produced.

The acetic acid fermentation in the method of producing of the present invention may be performed in the same manner as the conventional method of producing vinegar by a method of fermentation by acetic acid bacteria. As for the medium used in acetic acid fermentation, it may be either synthetic medium or natural medium as long as they are containing carbon source, nitrogen source, inorganic substance and ethanol, and containing suitable amount of source of nutrition required by the used bacteria strain for its growth if it is needed.

As for carbon sources, various kinds of carbohydrates including glucose, sucrose and various kinds of organic acids, can be exemplified. As for nitrogen source, natural nitrogen source such as peptone, degradation product of the microorganisms and the like can be exemplified.

In addition, static culture, shaking culture, aeration-agitation culture and the like under aerobic condition are carried out, generally at the culture temperature of 30°C. The pH of medium is generally within the range of 2.5-7, preferably within the range of 2.7-6.5, and it also can be adjusted with various kinds of acids,

various kinds of bases, buffers and the like. Generally, after cultivation of 1 to 21 days acetic acid is accumulated at a high concentration in the medium.

(4) Embodiment of the present invention

Further, as recombinant plasmids pUSPT and pUSPT2 which are made by inserting ORF relating to the present invention or acetic acid resistance-gene comprising thereof (SEQ. ID No. 1 or SEQ. ID No. 3) into Escherichia coli vector (multi-copy vector) pUC19, i.e. pUSPT has been deposited as FERM BP-7932 with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan since March 1, 2002, and pUSPT2 has been deposited as FERM BP-8304 with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan since February 26, 2003, so that DNA relating to the present invention can be available without difficulty, and a person skilled in the art would easily carry out the present invention. In addition, if it is desired, by using these recombinant plasmids, ORF relating to the present invention or acetic acid resistance-genes comprising thereof, are transferred to the vectors capable of replicating autonomously in acetic acid bacteria, and these are introduced into acetic acid bacteria and cultivated. By this procedure, vinegar having high acetic acid content, can be easily produced.

Still further, as explained above and as it is also apparent from after-mentioned examples, deposit of source of acetic acid resistance-genes, embodiment of PCR,

preparation of plasmid vectors and recombinant plasmids, deposit of host bacteria and the like, have been elucidated, and each of them can be obtained, operated and processed easily, therefore, performance of each operation and treatment according to Examples leads to obtainment of the objected acetic acid tolerance-transformants. The use of them enables to produce acetic acid at a high concentration. Consequently, the present invention can be easily carried out in this point of view, as well.

The present invention will be explained more specifically with examples below.

Example

(Example 1) Cloning of acetic acid resistance-genes from *Gluconacetobacter entanii* and determination of the nucleotide sequence and the deduced amino acid sequence.

(1) Generation of chromosomal DNA library

Acetbacter altoacetigenes MH-24 strain (FERM BP-491), that is one strain of *Gluconacetobacter entanii* was performed shaking culture at 30° C on YPG medium (3% glucose, 0.5% yeast extract, and 0.2% polypeptone) added 6% acetic acid and 4% ethanol. After the cultivation, culture medium was centrifuged (7,500 × g, 10 min) and bacterial cells were obtained. Chromosomal DNA was prepared from the obtained bacterial cells by the method disclosed in patent literature 3.

The obtained chromosomal DNA as explained above was partially digested by restriction enzyme PstI (Takara Shuzo Co., LTD.), further the *Escherichia coli*-acetic acid

bacteria shuttle vector pMV24 was completely digested by restriction enzyme PstI. These types of DNA were mixed by adequate dose, ligated by using ligation kit (TaKaRa DNA Ligation Kit Ver. 2, Takara Shuzo Co., LTD.), and chromosomal DNA library of *Gluconacetobacter entanii* was constructed.

(2) Cloning of acetic acid resistance-genes

The chromosomal DNA library of *Gluconacetobacter entanii* obtained as explained above, was transformed into *Acetobacter aceti* No. 1023 strain (FERM BP-2287) that generally can grow only under up to approximately under 1% acetic acid concentration on agar medium.

Subsequently, transformed *Acetobacter aceti* No. 1023 strain was cultured for 4 days at 30°C on YPG agar medium containing 2% acetic acid and 100 µg/ml of ampicillin.

When colonies generated on the agar medium were inoculated into YPG medium containing 100 µg/ml of ampicillin and cultured, and plasmids were recovered from the obtained bacterial cells, the plasmid in which PstI fragment of approximately 4 kbp was inserted, could be recovered as shown in Figure 1 and this plasmid was named pP1. Further, it was confirmed that DNA fragment which enable *Acetobacter aceti* No. 1023 strain to grow on YPG agar medium containing 2% acetic acid, was approximately 2 kbp of EcoRV-BalI fragment in approximately 4 kbp of PstI fragment cloned into pP1.

The acetic acid resistance-gene fragment that enable to grow *Acetobacter aceti* No. 1023 strain on the agar medium containing 2% acetic acid, which strain usually can only be grown on agar medium containing up to approximately 1% acetic acid concentration, was thus obtained.

(3) Determination of the nucleotide sequence of cloned DNA

fragment

The cloned EcoRV-BalI fragment mentioned above was inserted into SmaI site of pUC19, and the nucleotide sequence of the fragment was determined by Sanger's dideoxy chain-termination method, and the nucleotide sequence described in SEQ. ID No. 1 was determined consequently. Sequencing was conducted in the all domains of the both strands of DNA, and it was also conducted so that cleavage sites were overlapped.

The presence of open reading frame (ORF) encoding 400 of amino acids (Figure 3), as shown in SEQ. ID No. 2, was confirmed in nucleotides 187 to 1386 of SEQ. ID No. 1.

(Example 2) Enhancement of acetic acid tolerance in transformant, transformed with acetic acid resistance-gene derived from *Gluconacetobacter entanii*

(1) Transformation of *Acetobacter aceti*

Acetic acid resistance-gene derived from *Acetobacter altoacetigenes* MH-24 strain (FERM BP-491) cloned as explained above, was amplified by PCR method with KOD-Plus- (TOYOBO Co., LTD.), and plasmid pSPT in which the amplified DNA fragment was inserted into restriction enzyme SmaI cleavage site of the acetic acid bacteria-*Escherichia coli* shuttle vector pMV24 (see non-patent literature 3, for instance), was constructed. The outline of amplified fragment inserted into pSPT is shown in Figure 1.

PCR method was performed as follows; i.e. PCR method was performed using genomic DNA derived from above-mentioned acetic acid bacterium as template and using primer 1 (the nucleotide sequence thereof is shown in SEQ. ID No. 5 (Figure 7)) and primer 2 (the nucleotide sequence thereof is shown

in SEQ. ID No. 6 (Figure 8)) as primer in the following condition.

PCR method was performed i. e. for 30 cycles, in which 1 cycle is consisting of performance at 94°C for 15 sec, 60°C for 30 sec, and 68°C for 2 min.

This pSPT was transformed into *Acetobacter aceti* No. 1023 strain by electroporation method (see non-patent literature 6, for instance). The transformants were selected on YPG agar medium added 100 µg/ml of ampicillin and 2% acetic acid.

Plasmid was extracted from the transformant having ampicillin resistance grown on the selection medium in the conventional manner for analysis and it was confirmed that it retained the plasmid having acetic acid resistance-gene.

(2) Acetic acid tolerance of the transformant

The ampicillin-resistant transformant having plasmid pSPT obtained as explained above was compared their growth on YPG medium added acetic acid to that of the original strain of *Acetobacter aceti* No. 1023 strain introduced only shuttle vector pMV24.

Specifically, the transformant including pSPT and the original strain having shuttle vector pMV24 were inoculated into 100 ml of YPG medium containing 3% ethanol and 100 µg/ml of ampicillin and into 100 ml of YPG medium containing 3% ethanol, 3% acetic acid and 100 µg/ml of ampicillin, respectively, and shaking culture (150 rpm) was performed at 30°C, and growth of the transformant in the medium containing acetic acid was compared to that of the original strain by measuring absorbance at 660 nm.

Consequently, it was confirmed that the transformant

and the original strain *Acetobacter aceti* No. 1023 could grow almost similarly in the medium without containing acetic acid, while the transformant could grow but the original strain could not grow in the medium containing 3% acetic acid and 3% ethanol, as shown in Figure 2. Function of enhancing acetic acid tolerance of acetic acid resistance-gene was confirmed.

(3) Thermal resistance of the transformant

The ampicillin-resistant transformant having plasmid pSPT obtained in above-mentioned (1), was compared their growth on YPG medium in which the cultivation temperature was changed, to that of the original strain of *Acetobacter aceti* No. 1023 introduced only shuttle vector pMV24.

Specifically, they were cultured with agitation rate at 400 rpm, aeration rate at 0.2 vvm and temperature of 30°C in 1 L of YPG medium containing 1% acetic acid, 4% ethanol, and 100 µg/ml of ampicillin using 2 L of mini-jar fermenter (Chiyoda Seisakusho Co., LTD.: TBR-2-1), and fermented up to approximately 3% acetic acid concentration. Then, the culture medium was withdrawn from the mini-jar fermenter with 200 ml of it left, 800 ml of YPG medium containing acetic acid, ethanol and 100 µg/ml of ampicillin was newly added, it was adjusted to the concentration of 4% ethanol and 1% acetic acid, culture temperature was risen to 33°C, and fermentation was thus restarted.

When fermentation further progressed and concentration of acetic acid in the medium became approximately 3%, the culture medium was withdrawn again and the medium was added again, further culture temperature was risen to 36°C to ferment them similarly, and still further

acetic acid fermentation was performed by raising temperature 1°C at a time in the same manner.

Then, growth of bacteria was compared by measuring absorbance at 660 nm, and the ratio of acetic acid fermentation was compared by measuring acetic acid concentration in culture medium.

As a result, acetic acid fermentation and growth of bacteria at 40°C were possible in the transformant, while acetic acid fermentation and growth of bacteria could be confirmed only up to 37°C in the original strain of *Acetobacter aceti* No. 1023 as shown in figure 3, and the function of enhancing acetic acid tolerance of SPT gene was confirmed.

(Example 3) Acetic acid fermentation of the transformant transformed with acetic acid resistance-gene derived from *Gluconacetobacter entanii*

The ampicillin-resistant transformant having plasmid pSPT obtained in Example 2 was compared with fermentation ability to that of the original strain of *Acetobacter aceti* No. 1023 having only shuttle vector pMV24.

Specifically, they were cultured with agitation rate at 400 rpm, aeration rate at 0.20 vvm and temperature of 30°C in 2.5 L of YPG medium containing 1% acetic acid, 4% ethanol and 100 µg/ml of ampicillin using 5L of mini-jar fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-5A), and fermented up to 3% acetic acid concentration. Then, the culture medium was withdrawn from the mini-jar fermenter with 700 ml of it left, 1.8 L of YPG medium containing 100 µg/ml of ampicillin was newly added, it was adjusted to the

concentration of 4% ethanol and 1% acetic acid, and fermentation was thus restarted. The concentration of ethanol in the medium was maintained at 1% by addition of ethanol during the fermentation, and still further acetic acid fermentation was performed. Then, acetic acid fermentation ability of the transformant was compared to that of the original strain. The result was summarized in Table 1.

Table 1

	Final acetic acid concentra- tion (%)	Specific growth rate (OD660/hr)	Producti- on rate (%/hr)	Growth lag-time (hr)
Original strain	9.5	0.0151	0.103	62.5
Transfor- -mant	11.1	0.0323	0.136	24.0

From the result of Table 1, it was confirmed that the transformant was apparently superior in any of final acetic acid concentration, specific growth rate, production rate, and growth lag-time.

(Example 4) Enhancement of acetic acid tolerance in the transformant transformed with acetic acid resistance-gene derived from *Gluconacetobacter entanii*

(1) Transformation of *Acetobacter altoacetigenes*

Plasmid pSPT obtained in Example 2 was transformed into *Acetobacter altoacetigenes* MH-24 strain (FERM BP-491) which was one strain of *Gluconacetobacter entanii* by electroporation method (see non-patent literature 6, for

instance). The transformant was selected on YPG agar medium containing 0.55% agar added 100 µg/ml of ampicillin, 4% acetic acid and 4% ethanol.

The ampicillin-resistant transformant grown on the selection medium was extracted plasmid in the conventional manner for analysis and it was confirmed that they retained the plasmid having SPT gene.

Specifically, they were cultured with agitation rate at 500 rpm, aeration rate at 0.20 vvm and temperature of 30°C in 2.5 L of YPG medium containing 4% acetic acid, 4% ethanol, and 100 µg/ml of ampicillin using 5 L of mini-jar fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-5A), and fermented up to 6.3% acetic acid concentration. Then, the culture medium was withdrawn from the mini-jar fermenter with 700 ml of it left, 1.8 L of YPG medium containing 100 µg/ml of ampicillin was newly added, it was adjusted to the concentration of 6% and 4% ethanol, and fermentation was thus restarted. The concentration of ethanol in the medium was maintained at 1% by addition of ethanol during the fermentation, and still further acetic acid fermentation was performed. Then acetic acid fermentation ability of the transformant was compared to that of the original strain. The result was summarized in Table 2.

Table 2

	Final acetic acid concentration (%)	Specific growth rate (OD660/hr)	Production rate (%/hr)
Original strain	14.6	0.501	0.142
Transformant	16.2	0.756	0.175

From the result of Table 2, it was confirmed that transformants were apparently superior in any of final acetic acid concentration, specific growth rate, production rate, and growth lag-time.

(Example 5) Cloning of acetic acid resistance-gene from *Acetobacter aceti* and determination of the nucleotide sequence and the deduced amino acid sequence

Acetobacter aceti No. 1023 strain (FERM BP-2287) was performed shaking culture on YPG medium (3% glucose, 0.5% yeast extract and 0.2% polypeptone) at 30°C for 24 h. After the cultivation, culture medium was centrifuged (7,500 × g, 10 min) and bacterial cells were obtained. Chromosomal DNA was prepared from obtained bacterial cells by chromosomal DNA preparation method (see patent literature 3, for instance).

Cloning was conducted by inverse PCR method using above-prepared DNA as a template. Specifically, primer 3 (the nucleotide sequence thereof is shown in SEQ. ID No. 7 (Figure 9)) and primer 4 (the nucleotide sequence thereof is shown in SEQ. ID No. 8 (Figure 10)) were synthesized from domains which were thought to have higher conservation in comparison with other species from DNA sequence (SEQ. ID No. 1) obtained in *Acetobacter altoacetigenes* MH-24 strain. Next, PCR reaction was carried out using chromosomal DNA of *Acetobacter aceti* No. 1023 strain as a template, and approximately 750 bp of amplified fragments were obtained. Then, chromosomal DNA of *Acetobacter aceti* No. 1023 strain was completely digested by restriction enzyme PstI, and ligations were performed in the conventional manner. PCR

was performed using primer 5 (the nucleotide sequence thereof is shown in SEQ. ID No. 9 (Figure 11)) and primer 6 (the nucleotide sequence thereof is shown in SEQ. ID No. 10 (Figure 12)) and using the ligation product as a template and approximately 3 kbp of amplified fragment was obtained.

As a result of sequencing the nucleotide sequences of this fragment by Sanger's dideoxy chain-termination method using above-mentioned primers, the nucleotide sequence shown in SEQ. ID No. 3 was determined. Sequencing was conducted in the all domains of the both of strands of DNA.

(Example 6) Enhancement of acetic acid tolerance in the transformant transformed with acetic acid resistance-gene derived from *Acetobacter acetii*

(1) Transformation of *Acetobacter altoacetigenes*

Plasmid pSPT2 obtained in Example 5 were transformed into *Acetobacter altoacetigenes* MH-24 strain (FERM BP-491) which was one strain of *Gluconacetobacter entanii* by electroporation method (see non-patent literature 6, for instance). The transformants were selected on YPG agar media containing 0.55% agar added 100 µg/ml of ampicillin, 4% acetic acid and 4% ethanol.

The transformants having ampicillin tolerance, grown on the selection medium, were extracted plasmids in the conventional manner for analysis and it was confirmed that they retained the plasmids including SPT gene.

Specifically, they were cultured with agitation rate at 500 rpm, aeration rate at 0.20 vvm and temperature of 30°C in 2.5 L of YPG medium containing 4% acetic acid, 4% ethanol, and 100 µg/ml of ampicillin using 5 L of mini-jar

fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-5A), and fermented up to 6.3% acetic acid concentration. Then, the culture medium was withdrawn from the mini jar fermenter with 700 ml of it left, 1.8 L of YPG medium containing 100 µg/ml of ampicillin was newly added, it was adjusted to the concentration of 6% and 4% ethanol, and fermentation was thus restarted. The concentration of ethanol in the medium was maintained at 1% by addition of ethanol during the fermentation, and still further acetic acid fermentation was performed. Then, acetic acid fermentation ability of the transformant was compared to that of the original strain. The result was summarized in Table 3.

Table 3

	Final acetic acid concentration (%)	Specific growth rate (OD660/hr)	Production rate (%/hr)
Original strain	14.6	0.501	0.142
Transformant	16.0	0.605	0.153

From the result of Table 3, it was confirmed that the transformants were apparently superior in any of final acetic acid concentration, specific growth rate, production rate, and growth lag-time.

Industrial Applicability

According to the present invention, novel genes participating in acetic acid tolerance can be provided, further bred strain capable of efficiently producing vinegar at higher acetic acid concentration by using the genes can be obtained. Still further, the present invention enables

to furnish a method of efficiently producing vinegar at higher acetic acid concentration using the bred strain.